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Complete List of Authors:	Kollmann, Martin; Philipps-Universität Marburg, Biology - Animal Physiology Rupenthal, Anna; Philipps-Universität Marburg, Biology - Animal Physiology Neumann, Peter; University of Bern, Institute of Bee Health, Vetsuisse Faculty Huetteroth, Wolf; Philipps-Universität Marburg, Biology - Animal Physiology Schachtner, Joachim; Philipps-Universität Marburg, Biology - Animal Physiology
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Novel antennal lobe substructures revealed in the small hive beetle *Aethina tumida*

Martin Kollmann[1], Anna Lena Rupenthal[1], Peter Neumann[2], Wolf Huetteroth[1,3], Joachim Schachtner[1]

[1] Philipps-University Marburg, Department of Biology, Animal Physiology, Karl-von-Frisch-Str. 8, 35032 Marburg, Germany
[2] Institute of Bee Health, Vetsuisse Faculty, University of Bern, Bern, Switzerland
[3] Current address: University of Konstanz, Department of Biology, Neurobiology, 78457 Konstanz, Germany

Abstract

The small hive beetle, *Aethina tumida*, is an emerging pest ~~f~~or~~f~~ social ~~honey~~bee colonies. *A. tumida* shows a specialized live style for which olfaction seems to play a crucial role. To better understand the olfactory system of the beetle, we used immunohistochemistry and 3D reconstruction to analy~~s~~ze brain structures, especially the paired antennal lobes (AL) which represent the first integration centers for odor information in the insect brain. The basic neuroarchitecture of the *A. tumida* brain compares well to the typical beetle and insect brain. In comparison to other insects, the AL are relatively large in relationship to other brain areas, suggesting that olfaction is of major importance for the beetle. The AL of both sexes contain about 70 olfactory glomeruli with no obvious size differences of the glomeruli between sexes. Similar to all other insects including beetles, immunostaining with an antiserum against serotonin revealed a large cell, which projects from one AL to the contralateral AL to densely innervate all glomeruli. Immunostaining with an antiserum against tachykinin-related peptides (TKRP) revealed hitherto unknown structures in the AL. Small TKRP-immunoreactive spherical substructures are in both sexes evenly distributed among all glomeruli. The source for these immunoreactive islets is very likely a group of about 80 local AL interneurons. We offer two hypotheses on the function of such structures.

Keywords

olfactory system, neuropeptide, serotonin, insect, 3D reconstruction

Introduction

The small hive beetle *Aethina tumida* (Murray 1867, Coleoptera: Nitidulidae) is a parasite and scavenger of colonies of social bees (honeybees: *Apis mellifera*: cf. Neumann and Elzen, 2004; bumblebees: *Bombus impatiens*: Spiewok and Neumann, 2006; stingless bees: *Trigona carbonaria*: cf. Greco et al., 2010 and *Austroplebeia australis*: cf. Halcroft et al., 2011)~~honeybee colonies, *Apis mellifera*~~. Both, ~~l~~larvae and adults of the small hive beetle feed on pollen, honey and bee brood, leading to fermentation of the honey, devastation of the ~~honey~~combs ~~and extermination of the brood~~ often resulting in the full structural collapse of the entire nest (Lundie, 1940; Schmolke, 1974, Neumann and Elzen, 2004). In its native range in sub-Saharan Africa, *A. tumida* is a rather harmless parasite, mostly affecting weak and stressed colonies (Lundie, 1940; Hepburn and Radloff, 1998; Neumann and Elzen, 2004; Neumann and Ellis, 2008). ~~Recently~~However, the small hive beetle has become an invasive species. It was introduced into the USA (1996), Egypt (2000), Australia (2001) and into Europe twice (2004 and 2014, see Neumann and Ellis, 2008; Mutinelli et al., 2014) and now has ~~with~~ well-established new populations in North America and Australia (Neumann and Elzen, 2004; Neumann and Elzen, 2008). In these areas, the small hive beetle can be considered a significant pest of managed honeybees ~~developed to a major threat leading to massive loss of bee colonies~~ (Neumann and Elzen, 2004) and possibly of wild bees (Neumann, 2015).

To control this emerging pest, Neumann and Elzen (2004) speculated about the possibility of a small hive beetle pheromone, which could be used for trapping systems. Indeed, male-produced aggregation pheromones of other species in the family Nitidulidae are known from *Carpophilus obsoletus* and are used for pest control (Petroski et al., 1994). Today, a vast variety of insect pheromones (especially for ~~l~~Lepidoptera and ~~c~~Coleoptera) is known and used for trapping systems (www.pherobase.com). Although pheromone communication has not yet been demonstrated in the small hive beetle, it has been shown that *A. tumida* is highly attracted to volatiles emitted by adult honey bees (*A. mellifera*), bumble bees (*Bombus impatiens*), stored pollen, wax, brood and honey (Suazo et al., 2003; Graham et al., 2011; de Guzman et al., 2011). Furthermore, small hive beetles prefer to fly before or after dusk (Schmolke, 1974) suggesting that visual cues are less important than olfactory cues when it comes to locating beehives for mating. Altogether, understanding the olfactory system of *A. tumida* might be instrumental to control this pest.

In insects, olfactory information is detected by olfactory sensory neurons (OSNs) housed in the antenna and the maxillary palps (reviewed in Hansson and Stensmyr, 2011). They pass the information on to the neuronal network in the antennal lobes (AL), the first integration centers for odor information in the insect brain. Typically, AL are substructured in spheroidal

compartments, the olfactory glomeruli. OSNs which express the same odorant receptor converge onto the same glomerulus (Vosshall et al., 2000). Glomerulus number ranges between about 40 in Diptera up to several hundred in Hymenoptera (Schachtner et al., 2005; Mysore et al., 2009; Kuebler et al., 2010). In various orders of neopteran insects including Coleoptera, Dictyoptera, Diptera, Hymenoptera, and Lepidoptera sexual dimorphic glomeruli have been described (Kondoh et al., 2003; Kleineidam et al., 2005; Schachtner et al., 2005; Hu et al., 2011). Such glomerular dimorphism may have been evolved independently where it was needed, e.g. for long distance pheromone detection or for the detection of specific odors like host plant volatiles or trail pheromone (Hansson and Stensmyr, 2011). In the AL, olfactory information is processed by local interneurons (LN) and relayed to projection neurons (PN) that connect to other brain areas including the mushroom bodies (MB) or the lateral horn (LH). Additionally, the AL receives innervation from a few unique centrifugal neurons (CN) that provide efferent input from other brain areas (reviewed in Schachtner et al., 2005).

Antennal lobes across insect species contain a wide range of neuromediators including excitatory and inhibitory transmitters like acetylcholine and GABA (e.g. Bicker, 1999; Homberg, 2002; Schachtner et al., 2005; Berg et al., 2009; Fusca et al., 2015). In addition AL neurons contain neuromediators like biogenic amines, gaseous signaling molecules like NO and a large variety of neuropeptides suggesting important involvement for proper olfactory behavior (e.g. Schachtner et al., 2005; Berg et al., 2007; Utz et al., 2008; Carlsson et al., 2010; Binzer et al., 2014; Siju et al., 2014; Fusca et al., 2015). For example, in moths and flies serotonin (5HT) is able to modulate the sensitivity of odors and sex pheromones (Linn and Roelofs, 1986; Gatellier et al., 2004; Hill et al., 2003; Kloppenburg and Hildebrand, 1995, Dacks et al., 2009). Another example are the tachykinin-related neuropeptides (TKRP), controlling olfactory sensitivity and locomotor activity in the fruit fly *Drosophila melanogaster* (Ignell et al., 2009, Winther et al., 2006; Winther and Ignell, 2010).

Typically, neuromediators are distributed across all glomeruli of the AL (Schachtner et al. 2005; Carlsson et al., 2010; Binzer et al., 2014, Neupert et al., 2012, Siju et al., 2014). However, there are exceptions, in which just one or several glomeruli receive innervations by neurons that express specific neuromediators like serotonin in the ant (*Camponotus laevigatus;* Dacks et al., 2006), the neuropeptide RLRW in the mosquito (*Aedes aegypti;* Siju et al., 2014), the neuropeptide sNPF in the fly (*Drosophila melanogaster;* Carlsson et al., 2010), or serotonin and several neuropeptides in collembolans (Kollmann et al., 2011a).

The life cycle of *A. tumida* involves long distance dispersal to new food sources (Neumann et al. 2012), preferentially after dusk (cf. Neumann and Elzen 2004). Therefore ~~shows a specialized live style for which~~ olfaction seems to play a pivotal role for the adult beetles

(reviewed in Neumann and ~~Ellis~~Elzen, 200~~8~~4). Given that olfaction is that important for the behavior of the animal, the anatomy of the brain especially of the central olfactory pathway might very likely reflect this importance. We hypothesize that brain neuropils involved in processing of olfactory information should be enlarged in relationship to other brain areas. We also hypothesize that a sequential invasion of bee colonies as it was postulated by Elzen et al. (2000), with males first and females following could be reflected by specialized glomeruli e.g. sexual dimorphic glomeruli as described for several other insect species. In addition, we are looking for any specialization in the central olfactory pathway which could reflect the special life style of *A. tumida*.

Materials and Methods

Experimental animals

Adult *Aethina tumida* ~~has been~~were collected from ~~a testing ground of the Department of Zoology and Entomology of the University of Pretoria (Pretoria/Tshwane South Africa). Beetles were collected from~~ naturally infested colonies~~hives~~ of the African honeybee subspecies *Apis mellifera scutellata* ~~and *Apis mellifera capensis*~~ at the experimental farm of the Department of Zoology and Entomology, University of Pretoria, South Africa. After collection, the beetles were immediately sexed following a routine procedure (Neumann et al., 2013). According to European ~~safety codes~~legislation, import of liv~~inge~~ *A. tumida* to Germany is ~~problematical~~ illegal (Commission Decisions EC No 2003/881 and Commission Regulation (EC) N° 1398/2003). Therefore, beetles were decapitated in Pretoria and heads were fixed for 12 hours at 4 °C in 4% FA (formaldehyde FA, Roth, Karlsruhe, Germany) in PBS (phosphate-buffered saline, 0.01 M, pH 7.4). Heads were rinsed for 15 minutes in PBS and afterwards stored in PBS in glass vials in a customized cooling device and had been sent by express delivery to the Philipps University of Marburg (Marburg, Germany).

Immunohistochemistry

Primary antibodies. In the current study, we used antibodies against the synaptic vesicle protein synapsin, the biogenic amine serotonin, and an antiserum recognizing tachykinin-related peptides (summarized in table 1).

The monoclonal antibody from mouse against a fusion protein consisting of a glutathione-S-transferase and the first amino acids of the presynaptic vesicle protein synapsin I coded by its 5´-end (SYNORF1; 3C11, #151101) was used to selectively label neuropilar areas. It was used in combination with one additional primary antibody raised in rabbit. The synapsin antibody was kindly provided by Dr. Erich Buchner (University of Würzburg, Germany), was first described by Klagges et al. (1996), and used in many insect studies to label neuropilar

areas (e.g. Utz et al., 2008; Heuer et al., 2012; Binzer et al., 2014). The antibody was used at a dilution of 1:100.

The polyclonal antiserum against serotonin (5HT) was raised in rabbit against paraformaldehyde-coupled conjugates of BSA and 5HT (DiaSorin, Dietzenbach, Germany). Its specificity for the insect nervous system was shown in several studies (e.g. Dacks et al., 2006). It was used at a dilution of 1:5000.

The polyclonal TKRP antiserum was kindly provided by Dr. H. Agricola (University of Jena, Germany). It was raised in rabbits against locustatachykinin-2 (Lom-TK II, APLSGFYGVRamide) glutaraldehyde-conjugated to bovine thyroglobulin (Veenstra et al., 1995). The antiserum is known to detect tachykinin-related peptides (TKRPs, consensus sequence FXGXRamide) in other insects as well (e.g. Vitzthum and Homberg, 1998; Heuer et al., 2012; Binzer et al., 2014). In beetles, specificity for the anti-TKRP antiserum was so far confirmed in *Tribolium castaneum* by preabsorption of the antiserum with synthetic Lom-TK II (Binzer et al., 2014). In the current study, we used the anti-TKRP antiserum to reveal morphological structures of the brain of *A. tumida*. It was used at a dilution of 1:2000.

Secondary antibodies. Goat anti-mouse antibodies conjugated to Cy5 (GAM-Cy5) and goat anti-rabbit antibodies conjugated to Cy3 (GAR-Cy3) were used as secondary antibodies (each 1:300; Jackson ImmunoResearch, Westgrove, PA, USA).

Whole mount double immunostainings. Brains of *A. tumida* ~~collected in Pretoria (South Africa),~~ were dissected out of the head capsule, fixed overnight at 4 °C in 4% FA in PBS, followed by rinsing (4 x 10 min) with PBS at RT (room temperature) ~~and sent to Germany~~. Afterwards brains were preincubated for 2 days in PBT (PBS containing 0.3% Triton-X 100, Sigma Aldrich, Steinheim, Germany) with 5% NGS (normal goat serum; Jackson ImmunoResearch, Westgrove, USA). The primary antibody anti synapsin (1:100) was used in combination with the anti Lom-TK II (1:5000) or anti 5HT antiserum (1:2000) diluted in PBT with 1% NGS. Brains were incubated for two days at 4 °C. After rinsing (4 x 10 min) with PBT at RT, brains were incubated in secondary antibodies (GAM-Cy5 and GAR-Cy3; 1:300) in PBT with 1% NGS at 4 °C for two days in the dark. After rinsing (6 x 10 min) with PBT at RT and washing in distilled H_2O for 10 min, brains were dehydrated in an ascending alcohol series (30%, 50%, 70%, 90%, 95%, 2 x 100% ethanol, 5 min each). Followed by clearing the tissue in methyl salicylate (10 min; Merck, Darmstadt, Germany) the brains were finally mounted in resin (Permount, Fisher Scientific, Pittsburgh, PA, USA).

Data processing

Fluorescence was analyzed with a confocal laser scanning microscope (Leica TCS SP5 Microsystems, Leica, Wetzlar, Germany), with the object lenses 20x oil objective (HCX PL APO lambda blue 20x/ NA = 0.70 Imm UV, working distance: 260 µm; Leica); 40x oil objective (HCX PL APO lambda blue 40x/ NA = 1.25 Oil UV, working distance: 100 µm; Leica) und 63x glycerol objective (HCX PL APO 63x/ NA = 1.30 Glyc 21°C CS working distance: 260 µm; Leica). We scanned with a resolution of 1024 x 1024 or 512 x 512 pixels, a line average of 2, speed of 200 Hz, a digital zoom of 1-2 and z-steps varying from 0.5 to 1.0 µm for detailed scans and from 3.0 to 5.0 µm for overview scans.

Image segmentation, reconstruction, and visualization

For three-dimensional reconstructions, brain structures were labeled by using the segmentation editor and were reconstructed by using the polygonal surface model in AMIRA 5.2 (Visage Imaging, Berlin, Germany). Segmentation and reconstruction were performed according to previously published procedures (Kurylas et al., 2008; El Jundi et al., 2009). Standard color codes were used for the reconstructed neuropils (Brandt et al., 2005). For further global processing (i.e. contrast and brightness optimization) and final figure arrangements, snapshots were taken in AMIRA and subsequently processed in Corel Draw 13 (Corel Corporation, Ottawa, Ontario, CA). Diagrams generated with Excel XP (Microsoft Corporation, Redmond, WA, USA) were imported and revised in Corel Draw 13 without any further modification. For statistical analyses, we used two-tailed t-test in Origin 6.0 (OriginLab Corporation, Northampton, MA, USA) and Excel XP.

Results

General organization of the brain

A three-dimensional reconstruction of the brain of *Aethina tumida* was created based on confocal sections of an adult female stained with anti synapsin antibody (Fig. 1) (movie of a rotating 3D reconstruction and a camera path through the synapsin staining of a brain can be found in the digital supplements, Suppl. 1, 2). The brain contains all typical neuropils known from most insects including neuropils of the optic lobes, the antennal lobes, mushroom body and neuropils of the central complex (e.g. *Drosophila melanogaster*, Rein et al., 2002; honeybee *Apis mellifera*, Brandt et al., 2005; desert locust *Schistocerca gregaria*, Kurylas et al., 2008; sphinx moth *Manduca sexta*, el Jundi et al., 2009; red flour beetle *Tribolium castaneum*, Dreyer et al., 2010). We reconstructed all neuropils that were clearly identifiable and separable (8 paired and 3 unpaired neuropils).

In the optical lobes, we reconstructed the paired medulla (Me), lobula (Lo), lobula plate (LoP), and accessory medulla (aMe). In the center of the protocerebrum the central complex is located. Its reconstruction includes the unpaired upper and lower unit of the central body (CBU, CBL), the paired Noduli (No) and the dorso-posteriorly located unpaired protocerebral bridge (PB).

The paired mushrooms bodies are placed lateral to both sides of the central complex. We reconstructed calyx (Ca) and pedunculus (Pe) separately. The Pe contains the vertical lobe (vL) and medial lobe (mL). The Pe and the lobes are separated into an inner core region, which is densely stained with synapsin antibody (Fig. 1; non-transparent shaped part of the MB) and a less densely anti synapsin stained exterior region (Fig. 1; transparent shaped part of the MB).

Organization of the antennal lobe

In total, we analyzed 12 ALs of 7 males und 9 ALs of 5 females. Characteristically for insects (Schachtner et al., 2005), in *A. tumida* the AL are organized in small, spherical substructures, the olfactory glomeruli, which are arranged around a central coarse neuropil. The small hive beetle possesses ~~about 70 glomeruli. We found no sexual dimorphism in terms of glomeruli number. In detail, we counted~~ 72,~~.~~0±3.9 glomeruli per AL in males (n = 12 ALs~~; SD = 3,9~~) and 71~~,~~.1±3.4 glomeruli per AL in females (n = 9 ALs~~; SD = 3,4~~)(p = 0,588).

The average size of one glomerulus is 10.7~~10~~±1.8 µm³ in males (n = 12 ALs / n = 864 glomeruli~~; SD = 1.827 µm³~~) and 10.1~~40~~±1.8 µm³ in females (n = 9 ALs / n = 640 glomeruli~~; SD = 1.766 µm³~~). For ~~determination~~ approximation of the AL size, the volumes of all glomeruli within the AL were summed up~~.~~; ~~E~~extraglomerular space and central coarse neuropil were not ~~accounted for AL volume determination~~included. Mean volume of male AL is 7~~29~~30.~~975~~0±120.5 µm³ (n = 12 ALs~~; SD = 120.544 µm³~~), compared to 719~~,~~.2~~08~~±114.9 µm³ ~~(n = 9 ALs; SD = 114.883 µm³)~~ in females (n = 9 ALs). Taken together~~, we did not find a sexual dimorphism on the level of~~ male and female ALs of *A. tumida* were statistically indifferent in regard of glomeruli number (p = 0,588), overall glomeruli size (p = 0,875) or on the level of the AL volume (p = 0,997).

It is known from several insect species that in one of the sexes conspicuously larger glomeruli appear, typically at the entrance site of the antennal nerve (Schachtner et al., 2005; Hu et al., 2011). To verify whether this might be also true for the small hive beetle, we grouped the different-sized glomeruli of males (n = 12 ALs; n = 864 glomeruli) and females (n = 9 ALs; n = 640 glomeruli) according to their volume (0 – 999 µm³ = group 0; 1.000 – 1.999 µm³ = group 1; …35.000 – 35.999 µm³ = group 35), followed by analyzing the relative abundance of these different size groups (Fig. 2) (data of glomeruli from all ALs of one sex

had been pooled). No conspicuous sexual dimorphism could be seen. Middle-sized glomeruli with a volume between 4.000 µm³ and 11.999 µm³ seemed to be the most common ones, while small glomeruli (between 1.000 and 2.999 µm³) and large glomeruli (between 17.000 and 20.999 µm³) are less abundant. Even 2 to 5 larger glomeruli (between 21.000 and 35.999 µm³) occur in females as well as in males.

In summary, we found no evidence for any sexual dimorphism in the AL on the level of AL size, glomerulus number, and individual or overall glomerulus size.

Tachykinin-related peptides in the antennal lobes

Immunostaining with the anti TKRP antiserum in *A. tumida* revealed small, spherical substructures within all glomeruli (Fig. 3). In the anti synapsin staining these internal substructures are only slightly stronger labeled in comparison to the surrounding neuropil of the glomerulus (Fig. 5 b and b'). Each AL contains about 230 of these substructures (males: 245~~,.6~~7±14.3~~; mean of~~n = ~~3~~ ALs; ~~SD = 14,3 and~~ females: 224~~,.6~~±19.1~~,; mea~~n ~~= of~~ 5 ALs~~; SD = 19,13~~). We did not find a sexual dimorphism (p = 0.154). All observed glomeruli contain between one and ten substructures, which are never attached to the outer rim of a glomerulus but usually distribute evenly across the glomerular volume (Fig. 3). In both sexes the number of substructures in a glomerulus correlates almost perfect with the volume of the glomerulus (males: n = 3 ALs; n = 224 glomeruli; R^2 = 0,99; females: n = 5 ALs; n = 350 glomeruli; R^2 = 0,95 (Fig. 4).

The staining of TKRP-ir glomerular substructures originate most likely from a set of about 80±18 (n = 6 ALs~~; SD = 18~~) neurons located lateral in the AL (Fig. 3 e and e"; arrowheads), presumably exclusively local interneurons, which project their processes into the AL (3 e and e" arrows; see also electronic supplementary material movie S~~1~~3). As the antennal nerve shows no immunoreactivity to the TKRP antibody, we exclude that the staining of TKRP-ir glomerular substructures originates from OSNs. We also did not find any TKRP-ir fibers leaving the AL or entering the AL from other brain regions, excluding that the TKRP-ir glomerular substructures originate from projection neurons (PN) or centrifugal neurons (CN).

Serotonin in the antennal lobes

All glomeruli of one AL are innervated by axons branching from one, brightly stained main fiber, entering the AL at its medio-ventral side (Fig. 5 a; unfilled arrowheads). The origin of this 5HT-ir main fiber is most likely a single cell body, dorso-lateral to the AL at the contralateral side (as demonstrated in Fig. 5 a; arrow). The primary neurite runs through the AL without obvious branching and exits the AL at its dorsal side (Fig. 5 a; filled arrowheads). From here, the fiber runs dorsally and crosses to the contralateral hemisphere of the superior

protocerebrum where a divergent branch forms putative dendritic arborizations. The main fiber continues ventrally, to enter the contralateral AL (Fig. 5 a; unfilled arrowheads). The TKRP-ir glomerular substructures are not innervated by the 5HT-ir branches although some are touched just at their surface (Fig. 5 b, b' and b").

Discussion

General organization of the *A. tumida* brain

The overall anatomy of the brain of *A. tumida* (Fig. 1) compares well to other beetle brains regarding major neuropils including the optical lobes (OL), antennal lobes (AL), mushroom bodies (MB) and central body complex (CBX) (e.g. Van Haeften, 1993; Breidbach and Wegerhoff, 1994; Larsson et al., 2004; Dreyer et al., 2010; Hu et al. 2011).

The OL of the small hive beetle contain the paired medulla (Me), lobula (Lo), lobula plate (LoP), and accessory medulla (aMe). The LoP could be found so far only in Ephemeroptera, Trichoptera, Coleoptera, Lepidoptera, Diptera (Strausfeld, 2005), and Heteroptera (Settembrini and Villar, 2005). The AL consists of about 70 glomeruli, which seems to be a typical number for beetles; there are about 70 glomeruli in the red flour beetle *Tribolium castaneum* (Dreyer et al., 2010), about 60 glomeruli in scarab beetle *Holotrichia diomphalia* (Hu et al., 2011) and about 70 glomeruli in the cockchafer *Melolontha hippocastani* (third instar; Weissteiner et al., 2012). The AL will be discussed in more detail below. The paired MB contains the calyx (Ca) and the pedunculus (Pe) which is divided in the vertical and median lobe (vL and mL). The Pe, vL and mL can be separated in a densely synapsin stained core region and a less dense stained exterior region. This separation is in accordance to observations in the red flour beetle *Tribolium castaneum* (Zhao et al., 2008; Binzer et al., 2014) and ~~. It also resembles observations from~~ the African scarabid beetle *Pachnoda marginata*~~, where parts of the MB are separated into a central and an annular zone~~ (Larsson et al., 2004). The medial part of the right mL is overlapping the medial part of the left mL, as it has been observed in other beetles like *Tribolium castaneum* (Dreyer et al., 2010) or the blind cave beetle, *Neaphaenops tellkampfii* (Ghaffar et al., 1984). The unpaired central complex (CBX) can be separated into the protocerebral bridge (PB) and the central body (CB), which consists of the upper and lower unit (CBU and CBL), as well as the paired noduli (NO). This organization of the CBX is paralleled in other beetles like *Tribolium castaneum* (Dreyer et al., 2010) or the mealworm beetle *Tenebrio molitor* (Breidbach and Wegerhoff, 1994), as well as in many other insects (Homberg, 2008).

Comparison with relative brain neuropil volumes of other insects reveals that the AL of *A. tumida* are comparably large, only challenged by *T. castaneum* and the Madeira cockroach *Rhyparobia maderae* ~~(formally known as *Leucophaea maderae*)~~ (Tab. 2). The AL of the

small hive beetle take up about a fifth of the compared relative neuropil volumes, resembling the ratio found in *T. castaneum*, while the relative AL volume of *R. maderae* is even larger. *T. castaneum* is considered as an insect relying dominantly on olfactory cues (Dreyer et al., 2010), as. Also for are cockroaches (*Periplaneta americana*, Sakura and Mizunami, 2001) it had been shown, that based on their nocturnal behavior and an omnivorous feeding habit, they rely heavily on olfaction (Sakura and Mizunami, 2001), something, that is very likely also accounting for *R. maderae*. In summary, this result supports the hypothesis that for *A. tumida*, similar as stated for *T. castaneum*, olfactory cues are of major importance for their specialized behavior.

The relative volume of the MB are remarkably smaller in *A. tumida* (11,5 %) compared to *T. castaneum*, but still larger than in the majority of compared insects. MBs are higher integrative centers of the insect brain that are best known for their involvement in olfactory learning (e.g. McgGuire et al., 2001; Menzel, 2001; Heisenberg, 2003; Davis, 2004). However, insect MB are not solely higher centers of the olfactory pathway but are involved in the integration and processing of a broad range of sensory modalities including processing of visual, gustatory and mechanosensory information as well as contributions to sleep regulation, place memory, and temperature preference (reviewed in Heuer et al., 2012). Farris and Roberts (2005) demonstrated that generalist plant-feeding scarab beetles (Scarabaeidae), which belong to a generalist plant-feeding subfamily, have larger MB, while specialist dung-feeding scarab beetles have smaller MB. Interestingly, this difference in MB volume is independent of size and glomerulus number of the AL, the primary input olfactory neuropil of the MB. The small hive beetle and *Tribolium* are not part of the beetle family Scarabaeidae, but tThis observation may offer a possible explanation for the difference in MB volume between feeding specialist *A. tumida* and feeding generalist *T. castaneum*, which evolved as saprophytlic insects and naturally occur under the bark of trees, in rotten wood and infrequently in the nests of some Hymenoptera (Sokoloff, 1977; Grimm, 2001; Arnaud et al., 2005; Grimm, 2001; Sokoloff, 1977).

In *A. tumida*, the OLs are with about two third of the relative neuropil volume, larger than the OLs of *Tribolium*. In insects, larger OLs correlate primarily with larger complex eyes containing more photoreceptor cells. *Tribolium* has relative small compound eyes (80–83 ommatidia per eye; Friedrich et al., 1996) compared to other insect species including the small hive beetle.

The role of the central body is probably best described as a central coordinating functionor in sensory and motor integration (for reviews see Strauss, 2002; Wessnitzer and Webb, 2006; Homberg, 2008). With 4,8 %, the relative volume of the central body of *A. tumida* is smaller than the same structure in *Tribolium* and *Heliothis virescens* but still larger than in the fly,

honey bee, locust, cockroach or ~~2~~two Lepidoptera species. This suggests for the two beetles a more prominent~~complex~~ function of the central complex than in most other insects. In this context, it would be interesting to have more comparable central complex volumes of other Coleoptera with different lifestyles e.g. water beetles or non-flying beetles.

Olfactory driven behavior and sexual dimorphism

Males of the related beetle *Carpophilus obsoletus* release an aggregation pheromone that attracts both sexes (Petroski et al., 1994) leading to the hypothesisthat a similar pheromone could guide *A. tumida* into host beehives that have been parasitized already (Elzen, 2000; Neumann and Elzen, 2004). However, a sequential arrival of male and female *A. tumida* could not be observed (Spiewok and Neumann, 2012). This does not rule out sex-specific differences in olfaction; females seem to be more responsive to beehive volatiles than males (Suazo et al., 2003), and this might be reflected in a sexual dimorphism of the olfactory system of *A. tumida*.

~~Until recently it was suggested that male hive beetles enter a beehive before the females, leading to the speculation, that male *A. tumida* may distribute an aggregation pheromone attractive for both sexes (Elzen, 2000; Neumann and Elzen, 2004) as observed in *Carpophilus obsoletus* (Petroski et al., 1994). But pheromone communication has not yet been demonstrated in the small hive beetle. More recent studies question these earlier observations that males tend to infest before females and its resulting theory of such an aggregation pheromone (Spiewok and Neumann, 2012). Spiewok and Neumann (2012) could not find any evidence that males enter the beehive before females. Paradoxically, females seem to be even more responsive than males to the different beehive volatile sources (volatiles from adult worker bees, pollen, unripe honey, beeswax, wax by products, and bee brood) (Suazo et al., 2003). However, any possible use of a pheromone or different responses to beehive volatiles of males compared to females might be reflected in a sexual dimorphism of the olfactory system of *A. tumida*.~~

Sexual dimorphism has been described in various insect species on different levels of the olfactory pathway ranging from the periphery to the central nervous system including the antenna (e.g. beetles: Kaissling, 1971; Ågren, 1985; Allsopp, 1990; Renou et al., 1998; Diptera: Clements, 1999; Ruther et al., 2000; Stocker, 2001; Hymenoptera: Steinzer et al., 2013; moths: Schneider, 1992; Rospars and Hildebrand, 2000; Huetteroth and Schachtner, 2005), the specificity, number and/or distribution of olfactory receptors (e.g. moths: Miura et al., 2009; Nakagawa et al., 2005 or Diptera: Bohbot et al., 2007), the morphology and number of glomeruli in the AL (Kondoh et al., 2003; Schachtner et al., 2005; Hu et al., 2011; Kelber et al., 2010; Streinzer et al., 2013), and higher order brain structures (e.g. *Drosophila*:

Cachero et al., 2010). Detailed analyses of the antennae of *A. tumida* are missing. So far, there is no evidence that demonstrates a sexual dimorphism at the level of the antenna of the small hive beetle and. Iinformation on the distribution of olfactory receptors for beetles is rare. For *T. castaneum*, transcription analyses of female and male antenna show no sexual dimorphism in the expression of odorant receptorsORs (Dippel et al., in preparation).; Ddata for the small hive beetle are lacking so far.

Morphology of olfactory glomeruli

Differently sized sexual dimorphic glomeruli have been observed in a wide range of insects including beetles, cockroaches, bees, ants, moths, flies and mosquitoes (Schachtner et *al. 2005; Vosshall und Stocker 2007*; Hu et al., 2011). Typically these are one up to five glomeruli of a so-called "macroglomerular complex" in males to detect sex pheromones or in ants to detect trail pheromones, or "female sex specific glomeruli" to detect host plants for oviposition. Such glomeruli are normally positioned at the entrance area of the antennal nerve into the AL (Hansson, 1997; Anton and Homberg, 1999; Rospars and Hildebrand, 2000; Schachtner et al., 2005; Kleineidam et al., 2005). It is unclear whether the glomeruli of the "macroglomerular complex" and the "female sex specific glomeruli" are homologous or not (Rospars and Hildebrand, 2000). One prominent example are the moths, where males have between three and four macroglomeruli and the females between three and five sex specific glomeruli (reviewed in Schachtner et al., 2005). In cockroaches *Periplaneta americana* (Boeckh und Tolbert, 1993) and *Blaberus craniifer* (Rospars und Chambille 1981) males have one macroglomerulus while females lack any equivalent structure. In fruit flies, sexual dimorphism in AL glomeruli is anatomically less conspicuous (Fishilevich und Vosshall 2005, Vosshall und Stocker 2007), and homology to male macroglomeruli of other insects is not yet clear (Kondoh et al., 2003). A good example of a dominating sexual dimorphism is the AL of the male honeybee *Apis mellifera* with a macroglomerular complex, occupying 40% of the whole AL (Arnold et al., 1985). A recent studyHu et al (2011) demonstrated such a sexual dimorphism for the first time in a beetle, identifying a single macroglomerulus in the AL of the Korean black chafer (*Holotrichia diomphalia*; Hu et al. 2011). The current study addresses the AL morphology of the small hive beetle in detail, but neither male macroglomeruli nor female sex specific glomeruli could be found.

Number of glomeruli in the antennal lobes

A difference in number of glomeruli between sexes is common among insects. Yellow fever mosquito females have one additional glomerulus (Ignell et al. 2005), Pieris brassicae female butterflies have three glomeruli more than their male counterparts (Rospars 1983). In

hymenopterans, female honeybee workers possess about 160 glomeruli compared to about 106 glomeruli in males (Arnold et al., 1985; Flanagan and Mercer, 1989; Brockmann and Brückner, 2001). ~~A great disparity can be found in *A. mellifera* ALs. Female worker possess about 152 – 166 glomeruli (Arnold et al. 1985; Flanagan and Mercer 1989), while males have with 104 glomeruli about a third less glomeruli (Arnold et al., 1985; Brockmann and Brückner, 2001). In the yellow fever mosquito *Aedes aegyptii,* females have one additional 50$_{th}$ glomerulus (Ignell et al., 2005). In the white cabbage butterfly *Pieris brassicae* no macroglomerular complex or female sex specific glomeruli could be observed, but females possess with 63 glomeruli three glomeruli more than males (Rospars, 1983).~~ In beetles, varying glomerulus numbers between sexes have so far not been described. In *A. tumida* we found with about 70 glomeruli in both sexes no ~~significant difference between females and males~~sexual dimorphism. ~~Both sexes contain about 70 glomeruli which compares well to glomeruli numbers described in beetles to date: about 70 glomeruli in *T. castaneum* (Dreyer et al., 2010), about 60 glomeruli in *H. diomphalia* (Hu et al., 2011), and about 70 glomeruli in third instar larvae of *Melolontha hippocastani* (Weissteiner et al., 2012).~~

In summary, the absence of a sexual dimorphism in the olfactory pathway of *A. tumida* does not favor the work of Spiewok and Neumann (2012) or of Elzen (2004) and can on this level of analysis not add to an better understanding, why female *A. tumida* respond more strongly to beehive volatiles than males (Suazo et al., 2003). A detailed analysis of the *A. tumida* antenna including distribution of olfactory sensillae and identification and distribution of olfactory receptors would be necessary. Such insights could be helpful to create/optimize olfactory beetle traps.

Serotonin-ir neuron in the AL

The observed innervation of all glomeruli of one AL by only one 5HT-ir cell body is common among insects (Dacks et al., 2006) as well as for ancestral hexapods, as observed in collembolans (Kollmann et al., 2011a). The described anatomy of the 5HT-ir neuron has been described for Coleoptera before and can also be found in Lepidoptera, Trichoptera, non-Schizophoran Diptera and Neuroptera (Dacks et al., 2006) and is very likely also true for aphids (Kollmann et al., 2011b). A side branch of the 5HT-ir neuron into the ipsilateral lateral protocerebrum as observed in many insects (including Coleoptera; Dacks et al., 2006) could not be found in *A. tumida*.

Glomerular substructures

Immunostaining with an antiserum recognizing tachykinin-related peptides (TKRPs) resulted in the discovery of small, spherical substructures, which are evenly distributed among all glomeruli, with no obvious difference between both sexes. These substructures seem to

originate from a cluster of cell bodies, presumably local interneurons, lateral in the AL. Immunostaining with the same antiserum in two other beetles, *Tenebrio molitor* (Wegerhoff et al., 1996) and *T. castaneum* (Binzer et al., 2014), resulted in a cluster of local interneurons located in a similar position lateral in the AL. They provid~~inge~~ the glomeruli with a dense meshwork of projections but give not rise to spheroidal structures as described here. Like for *A. tumida*, the antiserum did not reveal fibers in the antennal nerve or fibers belonging to either projection or centrifugal neurons (Wegerhoff et al., 1996; Binzer et al., 2014). Similarly, in all other insects where TKRP stainings were performed, the antisera labeled only local interneurons in the AL and no other neuron types (Schachtner et al., 2005; Carlsson et al., 2010; Neupert et al., 2012; Binzer et al., 2014; Siju et al., 2014). In summary and in contrast to all other insects examined before, the TKRP positive local neurons provide each glomerulus with particular islet like projections.

In ALs of insects, all or only a subpopulation of glomeruli can be target of individual neurons or of populations of neurons and a variety of innervation pattern of olfactory glomeruli by AL neurons (LNs, PNs) or CNs has so far been described either by filling of single neurons or by immunostaining (summarized in Fig. 6, reviewed in Schachtner et al., 2005, Seki and Kanzaki, 2008; Husch et al., 2009; Carlsson et al., 2010; Chou et al., 2010; Seki et al., 2010; Neupert et al., 2012; Binzer et al., 2014; Siju et al., 2014). Comparing the different pattern of glomerulus supply via central neurons (CNs, LNs, PNs), innervation can ~~be~~occur a) only at the surface, b) scattered throughout the whole glomerulus c) or only through parts of the glomerulus, d) up to massive dense innervation of the whole or e) a distinct area of a glomerulus, or f) as described in this study, through islet like projections, which are evenly distributed throughout the glomerulus (Fig. 6). From our analysis, we cannot distinguish, whether an individual islet structure is supplied via a single axon or by more axons and whether islets of a single glomerulus are innervated by several or only by one neuron as suggested in figure 6f.

The role of LNs in the AL network is to shape the olfactory representation within and between the olfactory glomeruli to eventually form the output profile of the PNs via complex inhibitory and excitatory interactions (Stopfer et al., 1997; Sachse und Galizia, 2002; Wilson and Laurent, 2005; Olsen et al. 2007, 2010; Root et al., 2007; Shang et al., 2007; Silbering and Galizia, 2007; Olsen and Wilson, 2008; Okada et al., 2009; Tanaka et al., 2009; Chou et al., 2010; Seki et al., 2010; Wilson, 2013; Nagel et al., 2015). It is interesting that the small hive beetle developed, at least for a subpopulation of LNs, a morphologically different pattern of glomerulus innervation compared to other insects. With the available data, we can only speculate on the function of the islet like innervation.

We offer two hypotheses. Our first hypothesis argues that the islet like innervation is another effective way to provide the glomerular network with information carried by ~~the peptide~~ neuromediators e.g. neuropeptides. Such neuromediators~~Neuropeptides~~ can act as paracrine neurohormones, affecting a broad area surrounding the release site, also known as "volume transmission", in contrast to "wiring" (Agnati et al., 1995; Nässel, 2002). Considering the facts that the ~~amount of~~ islet~~s~~ number is linearly correlated to ~~the volume of a~~ glomerulus volume (Fig. 3) and that they are evenly distributed in each glomerulus, the islet~~s~~ contents could ~~be a peculiar way for a neuromediator to~~ affect the glomerulus network over prolonged periods of time or in an otherwise temporally unique fashion. ~~In this theory the islets of a glomerulus function as massive release sites with the capability to release vast amounts of the neuropeptide to supply either distinct sections or the whole glomerulus with this substance.~~

Our second hypothesis ~~goes a step further as we~~ interprets the islets as a specific adaptation of the beetles to their ~~live~~ lifestyle~~. This consideration sees the islets as part of a particular network~~ to cope with the complex chemical communication in a beehive with an olfactory system of a beetle. The TKRP immunostaining unmasks a group of LNs, which are part of such a particular network. It is known that chemical communication mainly based on pheromones is very important for bees (Slessor et al., 2005; Trhlin and Rajchard, 2011). To manage this olfactory task, worker bees have about 64,000 OSNs (Esslen and Kaissling, 1976), a large number of glomeruli (152–166 per AL in workers; Arnold et al., 1985), ~~only challenged by Formicoidea and Ichneumonoidea (Schachtner et al., 2005),~~ and about 170 ORs (Robertson and Wanner, 2006). For a parasitic insect living in a beehive survival and breeding success may highly depend on the ability to understand at least parts of the chemical communication of its host. As the repertoire of the beetle is restricted to about 70 glomeruli per AL, the islets could be part of a system ~~which~~ that allows the beetle to compensate for this disadvantage by expanding the glomerular coding space. ~~If we anticipate the islets as distinct subcompartments or subnetworks tuned to specifically decode bee communication, we would expect certain prerequisites. First, we would expect a defined group of neurons involved, which includes LNs but also PNs and possibly OSNs. With the LNs we have a defined group of about 80 cells which express at least a certain neuropeptide gene but very likely in addition a principal transmitter. In addition, specific PNs would be required that relay the extracted information to higher olfactory integration centers like mushroom bodies and lateral horn.~~ The genome sequence of another beetle, the red flour beetle *Tribolium castaneum*, revealed ~~far more~~a much higher number of functional ORs ~~in comparison to the number of~~ than olfactory glomeruli and it is still enigmatic what role these surplus ORs could play. The islets could be innervated by OSNs carrying different ORs than the OSNs that principally innervate the glomerulus. ~~Following this line, the islets would~~

~~functionally stand as specific "glomeruli" within the ordinary glomeruli and thus exaggerate the potential of the olfactory system of the beetle to cope with a more complex odor environment. A second prerequisite would be synaptic contacts between the neurons innervating the islets.~~ The synapsin immunostaining~~, which is indicative for chemical synapses,~~ is slightly stronger than in the surrounding parts of the glomeruli, suggesting a higher synaptic density within the islets. This argues for specialized zones with high synaptic communication between the involved neurons. If these islet like zones are targeted by specialized OSNs and/or PNs remains to be shown in the future~~Further studies are needed to prove our hypotheses~~.

Summary

Analyzing the brain of *A. tumida* by means of immunohistochemistry and 3D reconstruction revealed a basic brain neuroarchitecture comparable to other beetle and insect brains. In relationship to other brain areas and in comparison to other insects, the AL are ~~with a quarter of the compared neuropil volumes~~ relatively large, suggesting that olfaction is of major importance for the beetle. The AL of both sexes house about 70 glomeruli with no obvious size differences of the glomeruli between males and females. In accordance to what is typically found in other insects, staining with a 5HT antiserum revealed a large cell ~~which~~that projects from one AL to the contralateral AL to densely innervate all glomeruli. Immunostaining with an antiserum recognizing TKRPs revealed small spherical substructures, which are in both sexes evenly distributed among all olfactory glomeruli. The source for the TKRP-ir structures is very likely a group of about 80 local AL interneurons. The number of substructures ranges between one and ten and correlates linearly with the volume of the glomeruli. In total, one AL contains about 250 of these islets. For this unusual finding, we ~~have~~offer two hypotheses. ~~First, they are evenly distributed in the glomeruli and~~ 1) These evenly distributed substructures could act as massive releasing sites to deliver the neuro~~peptide~~mediator throughout the ~~surrounding~~ particular glomerulus. 2) ~~Another hypothesis sees t~~The islets act as ~~particularly~~ specialized subcompartments that expand ~~which exaggerate~~ the functional coding space~~ability~~ of the beetle's olfactory system.

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Legends

Tables

Table 1 List of antibodies used, including dilution, source, donor, and reference for each antibody

Table 2 Comparison of relative neuropil volume of *Aethina tumida* with eight different insect species including sex and sample number (*Drosophila melanogaster*, Rein et al., 2002; *Apis mellifera*, Brandt et al., 2005; *Schistocerca gregaria*, Kurylas et al., 2008; *Rhyparobia maderae*, Wei et al., 2010; *Manduca sexta*, el Jundi et al., 2009; *Godyris zavaleta*, Montgomery and Ott, 2014; *Heliothis virescens*, Kvello et al., 2009), *Tribolium castaneum*, Dreyer et al., 2010; and *Aethina tumida*, this work). With exception of the lobula plate, only neuropils with complements in all examined animals were compared (medulla, lobula complex, and lobula plate; antennal lobes, mushroom calyces and pedunculi, and the upper and lower unit of the central body).

Figures

Fig. 1 3D reconstruction of female brain of *A. tumida* in (a) anterior (b) dorsal and (c) posterior view. The neuropils were reconstructed with the AMIRA tools *SurfaceGen* and *SurfaceView*. The color code of the labeled neuropils is consistent with Brandt et al. (2005). AL, antennal lobe; Ca, Calyx; CBL, lower unit of the central body; CBU, upper unit of the central body; aMe, accessory medulla; Lo, lobula; LoP, lobula plate; Me, medulla; No, noduli; PB, protocerebral bridge; and Pe, pedunculus with lobes. Scale bar: 100 µm.

Fig. 2 Relative abundance of different sized glomeruli of both sexes. X-axis represents the different glomerular volumes, shown as volume groups (0 – 999 µm³ = group 0; 1.000 – 1.999 µm³ = group 1; …35.000 – 35.999 µm³ = group 35). The Y-axis represents the relative abundance in percent of the different size groups.

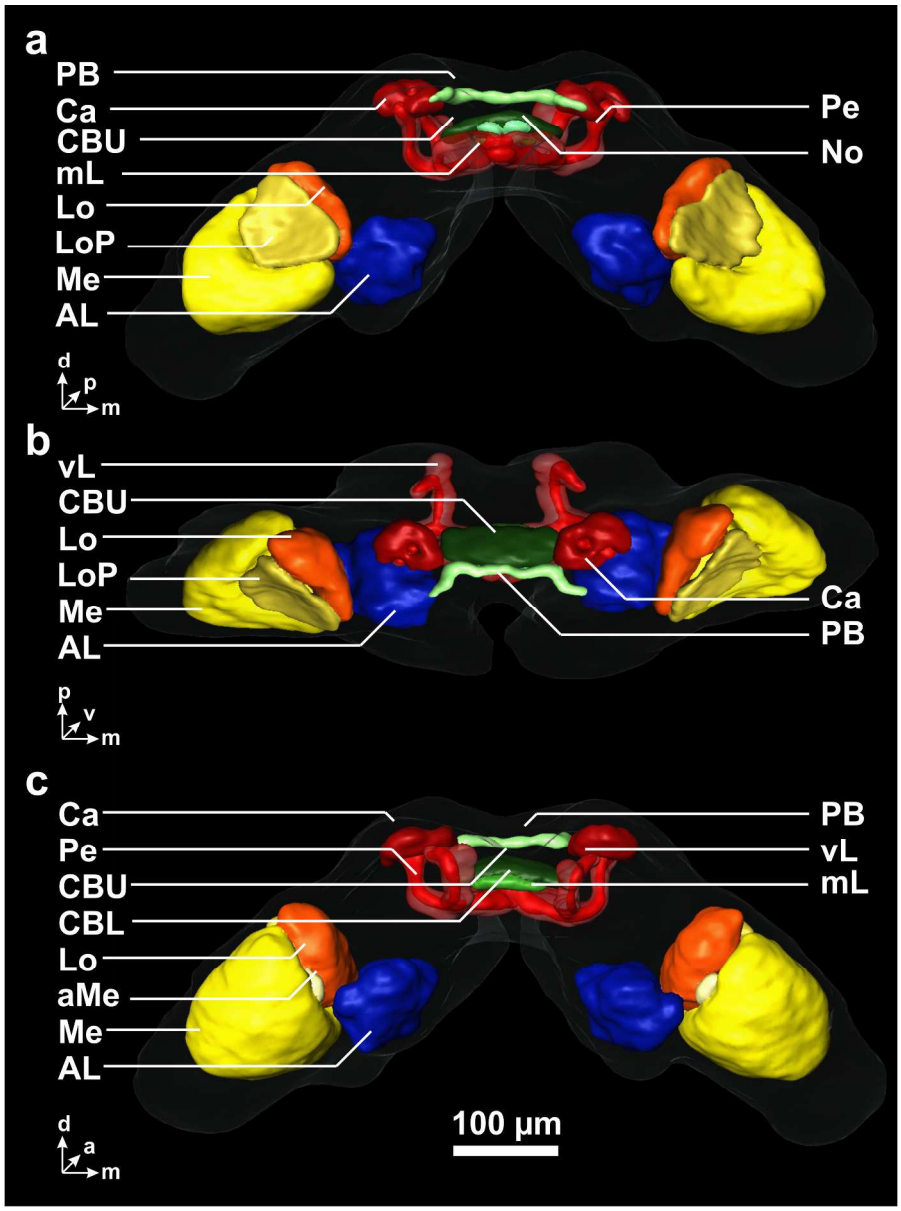
Fig. 3 The antennal lobe (AL) of *A. tumida* stained with synapsin (green) and Lom TKII (magenta). **a** Single optical section of an AL. Several individual glomeruli (G1 – G6), containing TKRP-ir glomerular substructures (dotted lines). **b** and **c** 3D reconstructions of glomerular substructures (b) and of the glomeruli (c) of the set of glomeruli outlined in A. **d** 3D reconstruction of the same set of glomeruli (transparent) (as in a) (G1- G6) including the glomerular substructures (gold colored). **e - e"** Staining with synapsin antibody (green in e; e') and TKRP antiserum (magenta in e; e") showing TKRP-ir local AL interneurons (arrowheads) and their axons (arrows) projecting into the core area of the AL (asterisk), from where they give rise to TKRP-ir substructures. e is an overlay of E' and E''. Boxed areas in E and E'' are enhanced in brightness and contrast to better demonstrate fibers of the local AL

neurons. Orientation bars in A valid for all subfigures: L = lateral, D = dorsal. Scale bars: 50 µm. Orientation bars: M = median, V = ventral. Scale bars: 50 µm.

Fig. 4 Abundance of glomerular substructures in relation to glomerulus size. The diagram shows a linear relationship between glomerulus size and number of glomerular substructures, with a coefficient of determination of 0,95 for male and 0,99 for female animals.

Fig. 5 Antennal lobe (AL) of *A. tumida* stained with anti synapsin (green) and anti serotonin (magenta) antibodies. **a** The maximum projection shows the branching of a single serotonin immunoreactive (5HT-ir) fiber, entering the AL at the dorsal site (unfilled arrowheads). Dorso-lateral to the AL, a single 5HT-ir cell body can be observed (arrow) projecting dorsal (filled arrowheads) without any branching or varicosities out of the AL. **b - b"** Single optical section of an AL. The glomerular substructures in the AL glomeruli are distinctly brighter stained with the synapsin antibody than the surrounding area of the glomeruli (b, b' dotted lines). 5HT-ir fibers clearly stay outside the glomerular substructures (b''). Orientation bars: M = median, V = ventral. Scale bars: 20 µm.

Fig. 6 Schematic drawing of principal innervation pattern of glomeruli**. a** Glomeruli are just sparsely innervated at the surface. **b** The innervation is scattered through the whole glomerulus. **c** The innervation is just scattered through a distinct area of the glomerulus. **d** The whole glomerulus is densely innervated by fine branches, which appears in immunohistological stainings as a bright, uniform staining. **e** A distinct area of the glomerulus is densely innervated by fine branches. **f** Branching appears just in several, small, distinct areas of the glomerulus (glomerular substructures), which are evenly distributed throughout the glomerulus.



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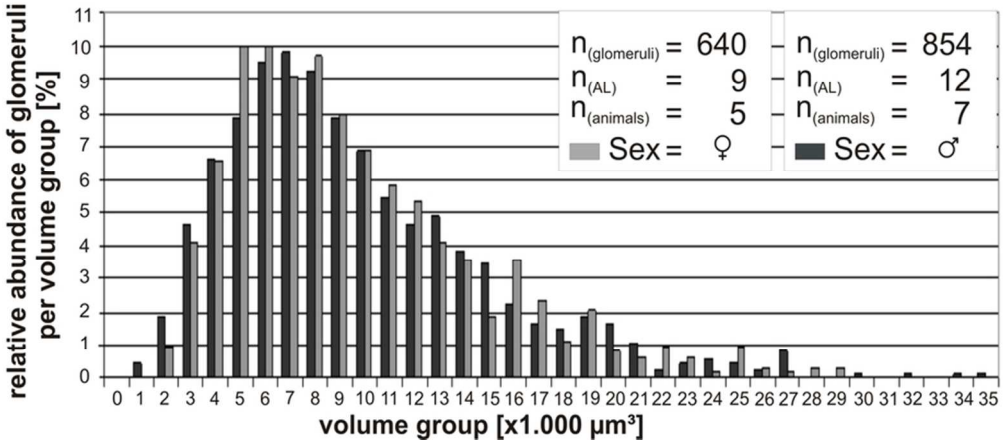

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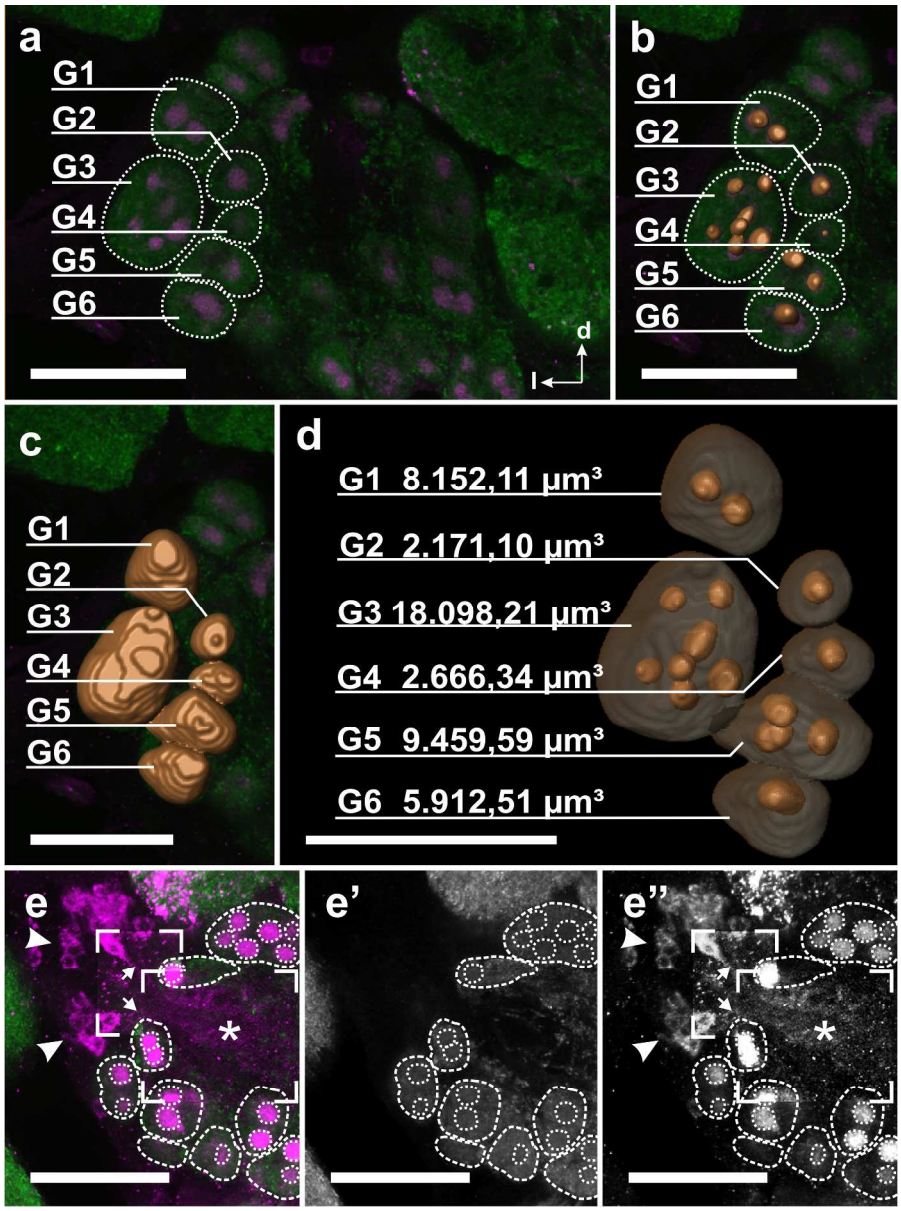

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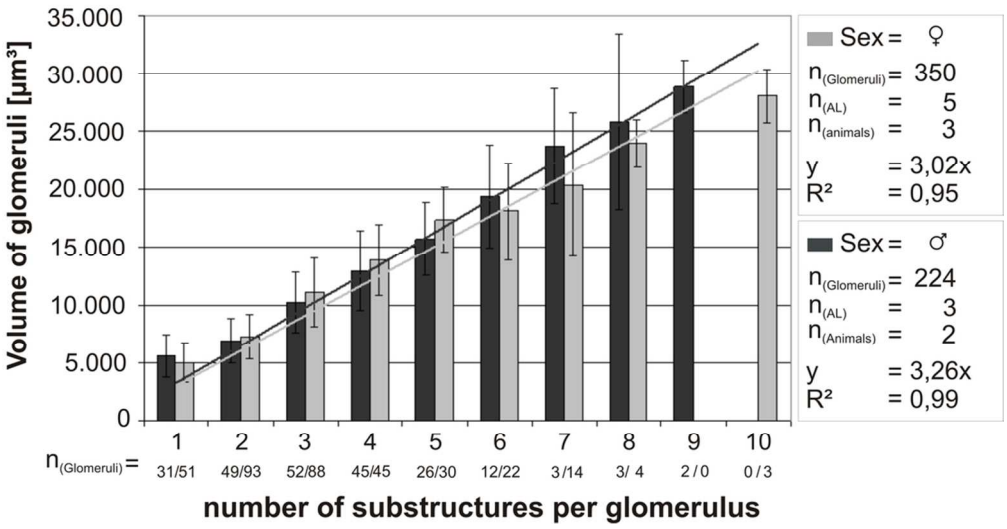

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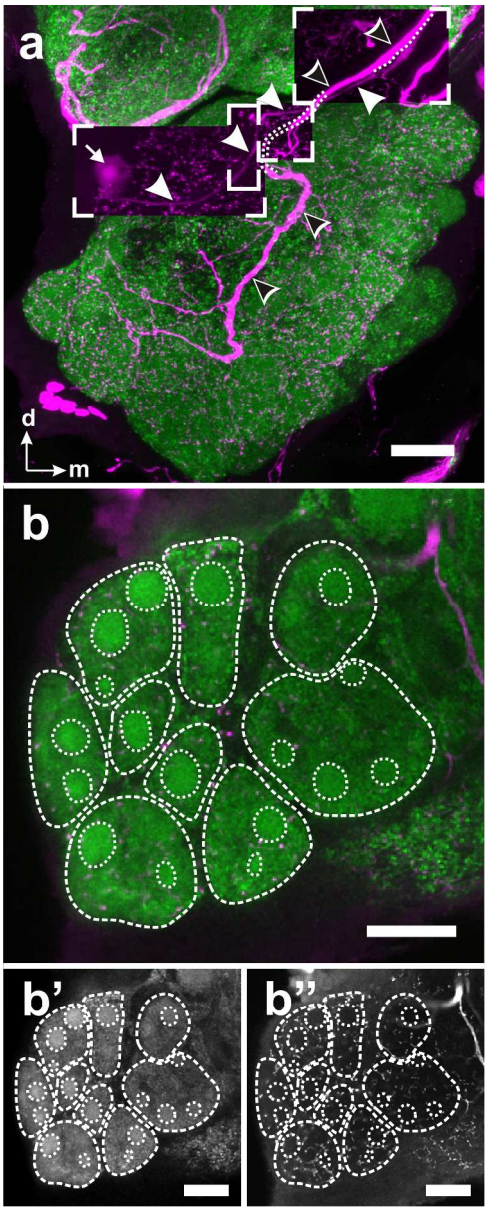

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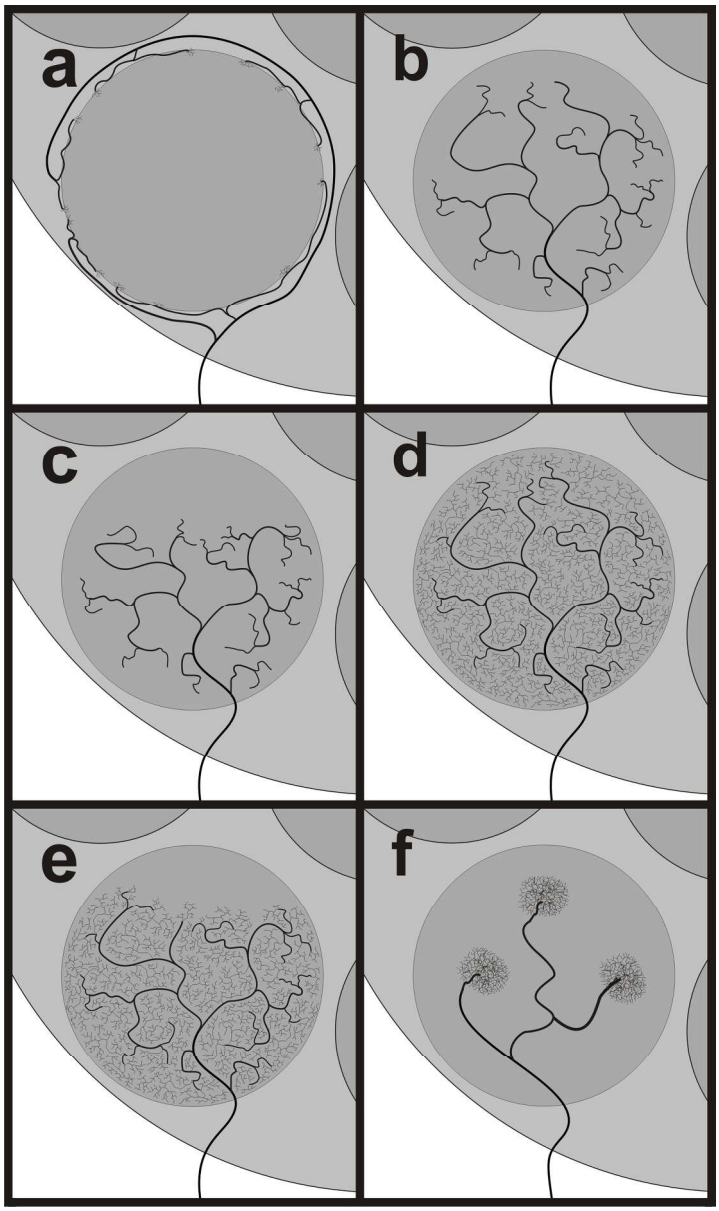

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141x238mm (300 x 300 DPI)

Name, anti-	Shortcut	Dilution	Source	Donor	Reference
D. melanogaster Synapsin I	α-Synapsin	1:100	Mouse	Dr. E. Buchner	Klagges et al., 1996
Serotonin	α-5HT	1:2000	Rabbit	DiaSorin	e.g. Dacks et al., 2006
Locusta migratoria Tachykinin II	α-TKRP	1:5000	Rabbit	Dr. H. Agricola	Veenstra et al., 1995

Table 1 List of antibodies used, including dilution, source, donor, and reference for each antibody

Order	Diptera	Hymenoptera	Orthoptera	Blattodea	Lepidoptera				Coleoptera		
Species	*D. melanogaster*	*A. mellifera*	*S. gregaria*	*R. maderae*	*M. sexta*		*G. zavaleta*	*H. virescens*	*T. castaneum*		*A. tumida*
Sex	♀	forager	♂	♂	♀	♂	♂/♀	♀	♀	♂	♀
N	28	20	10	20	12	12	8/8	10	20	20	1
AL (%)	9.5	8.5	9.7	35.6	12.8	15.0	8.7	15.7	22.4	24.5	21.6
MB (%)	7.5	32.7	16.0	37.2	6.9	6.8	6.1	13.6	21.9	23.8	11.5
OL (%)	79.6	57.9	72.6	23.0	79.4	77.3	84.4	64.5	50.1	45.1	62.1
CB (%)	3.4	0.9	1.7	4.2	0.9	0.9	0.8	6.1	5.6	6.6	4.8

Table 2 Comparison of relative neuropil volume of *Aethina tumida* with eight different insect species including sex and sample number (*Drosophila melanogaster*, Rein et al., 2002; *Apis mellifera*, Brandt et al., 2005; *Schistocerca gregaria*, Kurylas et al., 2008; *Rhyparobia maderae*, Wei et al., 2010; *Manduca sexta*, el Jundi et al., 2009; *Godyris zavaleta*, Montgomery and Ott, 2014; *Heliothis virescens*, Kvello et al., 2009), *Tribolium castaneum*, Dreyer et al., 2010; and *Aethina tumida*, this work). With exception of the lobula plate, only neuropils with complements in all examined animals were compared (medulla, lobula complex, and lobula plate; antennal lobes, mushroom calyces and pedunculi, and the upper and lower unit of the central body).